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1 **Identification and characterization of novel infection associated transcripts in**
2 **macrophages**

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9 Running title: Novel TLR4 and infection induced non coding transcript

10 Key words: Non coding transcript, TLR4, bacterial infection, THP1

11 **ABSTRACT:**

12 Regulated expression of genes in response to internal and external stimuli is primarily responsible
13 for the enormous plasticity and robustness of biological systems. Recent studies have elucidated
14 complex regulatory non protein coding transcript (lncRNA) circuits in coordinated response of
15 immune cells. By analysis of lncRNA expression profiles of macrophages in response to Mtb
16 infection, we identified novel highly expressed transcripts, unique in encompassing one functional
17 protein coding transcript- CMPK2 and a previously identified type I IFN responsive lncRNA- NRIR.
18 While these RNA are induced by virulent Mtb early, the complete absence of expression in non-
19 viable Mtb infected cells coupled to a more protracted expression profile in the case of BCG
20 suggest an important role in macrophage response to mycobacteria. Moreover, enhanced
21 expression was observed in macrophages from TB patients. The elevated expression by 1h in
22 response to fast growing bacteria further emphasizes the importance of these RNAs in the
23 macrophage infection response. These transcripts (TILT1, 2,3 - TLR4 and Infection induced Long
24 Transcript) are triggered exclusively by TLR4 stimulation (LPS) with faster and stronger kinetics

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25 in comparison to the lncRNA – NRIR. Overall, we provide evidence for the presence of numerous
26 transcripts that is a part of the early infection response program of macrophages.

27 Abbreviations: IFN- Interferon, NRIR, CMPK2

28 Introduction:

29 Intracellular bacteria encounter diverse host derived stresses and adapt to a wide ranging yet cell/
30 tissue specific niche(Huang and Brumell 2014; Hardison et al. 2018). Modulating the host to
31 subdue these potentially harmful responses is one of the strategies employed by pathogens for
32 long term infection (Cameron et al. 2015; Passalacqua et al. 2016). This plasticity requires an
33 enormous degree of flexibility in the pathogen's ability to not only sense the stimuli but fine tune
34 host transcriptional programs to suit survival inside the cells (Jimenez et al. 2016; Cornejo et al.
35 2017; Eisenreich et al. 2017). It is not surprising that gene expression patterns at the onset of
36 infection, immediate to the initial contact with host cells, often determine the outcome and overall
37 pathogenesis of the organism.

38 Over several years, a detailed coverage of expression has helped identify protein coding patterns
39 in diverse pathogens and cells/ intracellular niches in addition to paving the way for deciphering
40 the regulatory circuits of non-protein coding transcripts(Winchell et al. 2016; Hayward et al. 2018).
41 Recent evidences have delineated a definitive role for the long non-coding RNAs (lncRNA) in
42 orchestrating the immune response by regulating gene expression is now being recognized
43 (Imamura and Akimitsu 2014; Yu et al. 2015; Spurlock et al. 2016; Atianand et al. 2017). These
44 RNAs have been implicated in the regulation of several cellular processes, immune cell functions
45 and response to infections (Ouyang et al. 2014; Wang et al. 2014; Sigdel et al. 2015; Wang et al.
46 2015; Carpenter and Fitzgerald 2018; Xie et al. 2019; Menon and Hua 2020; Robinson et al.
47 2020). Non coding response dynamics have helped uncover novel facets of host – pathogen
48 interactions in several bacterial pathogens (Gomez et al. 2013; Wang et al. 2015; Roy et al. 2018;

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49 Yan et al. 2018). Not surprisingly, Mtb infection of macrophages induces several of the non-coding
50 transcripts with functional relevance in activating innate immune functions as well as modulation
51 of these responses by the pathogen (Yang et al. 2016; Huang et al. 2018; Sharbati et al. 2019).
52 Clinically relevant lncRNAs have recently been identified as putative biomarkers for TB patients
53 (Chen et al. 2017; Li et al. 2017; Wu et al. 2020). With phagocytes initiating a strong innate
54 immune response on primary contact with invading pathogens, it is logical to assume that
55 activation of surface TLRs would be one of the initial signals for expression of gene regulatory
56 networks in these cells. Recent advances in high throughput deep sequencing has revealed the
57 identity of novel transcripts that arise either out of novel ORFS, trans-splicing events, long
58 extensions and degradation products of previously identified transcripts (Srikumar et al. 2015;
59 Kalam et al. 2017; Jackson et al. 2018; Agirre et al. 2019). In our attempt to study the role of type
60 I IFN response in macrophages, we discovered that NRIR, previously identified as a lncRNA
61 regulating this pathway was one of the highest expressed non coding transcript in Mtb infected
62 macrophages. While the origins of these RNA is not yet clearly defined, detailed evaluation of this
63 genomic locus indicated the presence of multiple transcripts. In line with this, we identified 3 other
64 transcripts that appear to be encompassing NRIR and the neighboring IFN inducible gene –
65 CMPK2. We demonstrate that these transcripts- TILT 1,2,3 are induced in response to bacterial
66 infection temporally and is specific to activation of TLR4 by LPS. Expression of TILT is induced
67 significantly in patients with active TB. Early expression following stimulation of macrophages
68 suggests an important role for this transcript in the rapid response program of cells.

69 **Results:**

70 **The lncRNA NRIR is induced strongly in response to Mtb infection in macrophages:**

71 The response of macrophages to mycobacterial infection is characterized by an elaborate
72 expression of genes that include protein coding and non-coding transcripts. The type I IFN
73 signaling pathway is one of the early and robust response of host macrophage to mycobacterial

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74 infection (Novikov et al. 2011; Desvignes et al. 2012). Previous studies have identified the lncRNA
75 NRIR as a primary lncRNA regulating this response in infected cells (Kambara et al. 2014). We
76 hypothesized that NRIR, given the strong type I IFN signaling induced, would also be a crucial
77 component of the host response to Mtb in macrophages. In line with our surmise, NRIR showed
78 the highest expression levels in Mtb infected macrophages amongst a few of the well annotated
79 mammalian lncRNAs (Fig. 1A). While NRIR was expressed 6h post infection with a steady
80 increase in levels at later time points, the other innate response regulating lncRNA-NEAT1, was
81 more protracted and was expressed at ~3x higher by 24h of infection (Fig. 1B). In fact, NRIR
82 showed a steady rise in expression from the ~4x by 6h to a sharp increase by 24h of infection in
83 excess of 200 times the values in uninfected samples (Fig.1C). In response to a non-pathogenic
84 vaccine strain, BCG, NRIR was only induced by the later time point (24h) in infection, albeit at a
85 lower magnitude than Mtb infection (Fig. 1D). Interestingly, the response was completely
86 abrogated in macrophages exposed to heat killed Mtb even by 24h implicating an active infection
87 mediated induction of these non-coding transcripts in macrophages. The kinetic response of NRIR
88 induction observed in *S. typhimurium* and *E. coli* infections further validated the importance of
89 these transcripts in infection response of human macrophage. NRIR was visible by the 1st h of
90 infection followed by a steady increase of expression by 6h of infection in macrophages infected
91 by either strain of bacteria (Fig. 1E).

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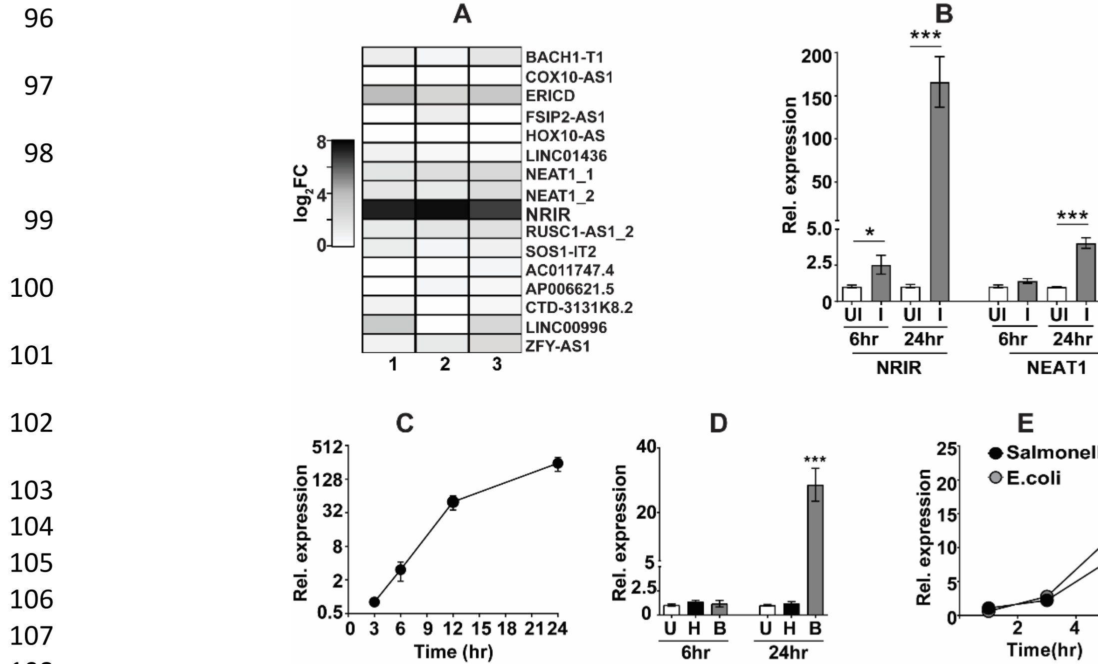

Figure 1: NRIR is expressed in response to bacterial infection.

(A) The expression levels of a few of the annotated non-coding transcripts was analysed by qRT PCR in THP1 macrophages infected with Mtb. Change in expression of triplicate assays relative to GAPDH ($\log_2$FC) is depicted as a heat map. Three independent replicate experiments are individually represented as mean expression values from triplicate assays (n=3).

B-E) Expression of NRIR (B-E) and Neat1 (B) in macrophages infected with Mtb (B-D) or *E. coli*/ or *S. typhimurium* (E) at different time intervals was analysed by qPCR. Change in expression of triplicate assays relative to GAPDH (logFC) is represented as rel. expression from triplicate assays of 2-3 independent replicate experiments. U- uninfected, H- Heat killed Mtb, B- *M.bovis* BCG.

The NRIR locus supports transcription of several other non-coding transcripts. NRIR is localized in the same genomic loci with the mitochondria associated gene – CMPK2 (Fig. 2A). While NRIR is the most abundant lncRNA, there have been reports of the presence of other non-

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125 coding transcripts in this locus ((Lagarde et al. 2017), Fig.2A). To evaluate other infection specific
126 transcripts, we used a PCR strategy with primers mapping to the NRIR/ CMPK2 specific region
127 (P1 and P4) and observed three distinct amplicons of ~400bp, 800bp and 1kb which were absent
128 when genomic DNA was used as a template (Fig. 2B). Both NRIR and CMPK2 specific amplicons
129 were observed in reactions with the larger 2 fragments as template (Fig. 2C). Moreover, while the
130 800bp fragment produced the 400bp and 800bp amplicons, the largest 1kb fragment again
131 yielded all 3 fragments on amplification with primers-1 and 4. Sequencing of the amplicons
132 revealed the presence of 3 unique splice variants with fusion of Exon 4 of CMPK2-203 and the
133 first exon of NRIR-202 (Fig. 2D). We then analyzed expression in total blood RNA of healthy
134 individuals. As seen in THP1 cells, amplicons of ~800bp and 1kb in PCR with specific primers 1
135 and 4 were observed confirming the universal presence of these transcripts (Fig. 2E).
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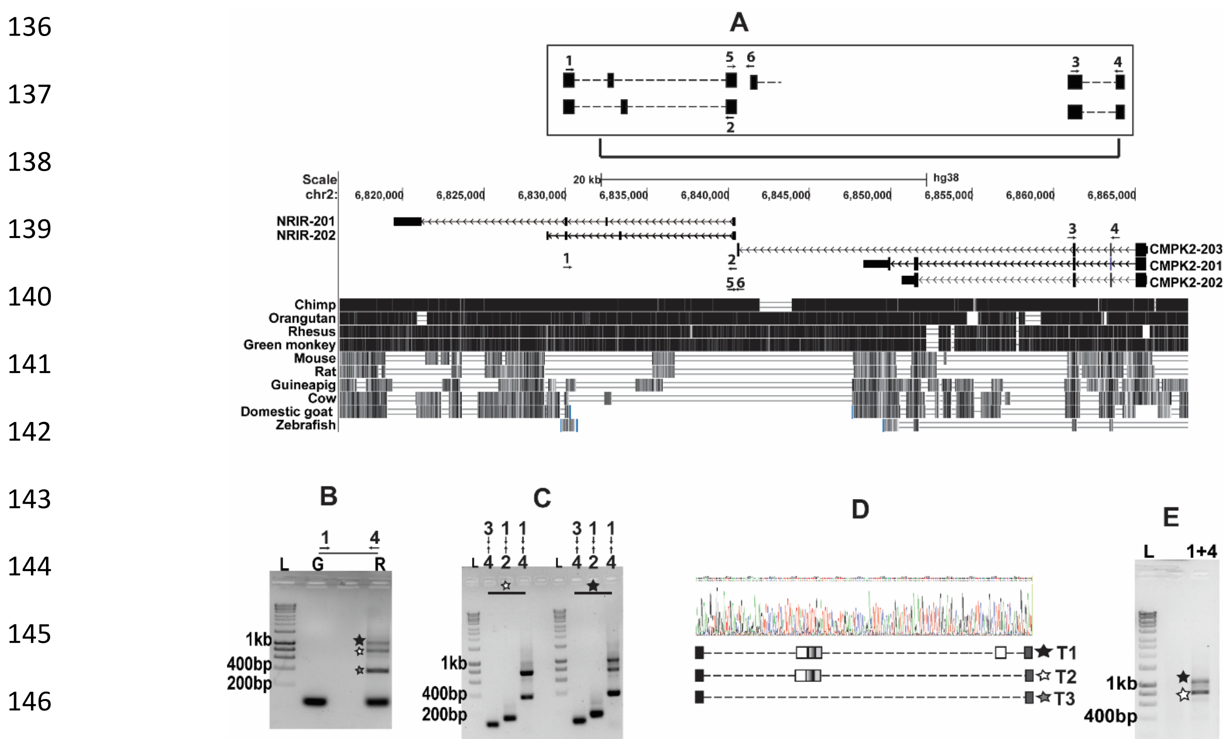

Fig. 2

149 **Fig. 2. Identification of the novel transcripts-TILT encompassing CMPK2 and another**
150 **lncRNA – NRIR in Mtb infected macrophages.**

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151 A- The genomic locus of CMPK2 and NRIR in human cells is depicted with the previously
152 annotated transcripts. The primers used for PCR analysis of transcripts are depicted by
153 numbers.
154 B, E- Analysis of the PCR amplification of cDNA (B, 24h) by using primers (1,4) from THP1
155 cells infected with Mtb as indicated to regions as depicted in panel A of the genomic locus by
156 agarose gel electrophoresis. C- Amplification of the PCR product of reaction in B with primers
157 as indicated in the figure. D- The profiles of exons present in the 3 new non-coding transcripts
158 identified by sequencing of the PCR products. E- Expression of the novel transcripts in total
159 blood of a healthy individual analysed by agarose gel electrophoresis.

160 **The novel transcripts are actively induced in response to bacterial infection of**
161 **macrophages**

162 Macrophages are typically designed to respond to assorted stimuli and alter their transcriptional
163 profile to suit the incoming insult. Being a critical component of the host innate defense,
164 macrophages are fine tuned for a rapid response to infections. TILT, in contrast to NRIR was
165 more protracted and reduced- expression in macrophages was seen only by 12h of infection
166 (4fold) with Mtb with a further increase to 14-fold by 24h (14-fold) (Fig. 3A). The absence of TILT
167 expression in macrophages infected with heat inactivated Mtb even by 24h argued for the
168 importance of active infection in the expression of TILT. Moreover, delayed expression of these
169 RNAs only by 24h in response to infection by a non-pathogenic slow growing mycobacterial strain-
170 *M. bovis* BCG further corroborated the importance in the response to pathogenic infection (inset).
171 Clinical relevance in infection was highlighted by the significantly enhanced expression in the
172 blood of active TB patients in comparison to healthy individuals (Fig. 3B). A similar profile of
173 enhanced expression of TILT was observed for infection with Salmonella and *E. coli* (Fig. 3D),
174 expression levels of TILT showed a strong peak (~25-40 fold) by 3h of infection followed by a

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175 rapid decline to around 10- fold greater than basal levels by 6h of infection associated with the
176 faster growth rate of these bacteria in comparison slow growing mycobacteria.
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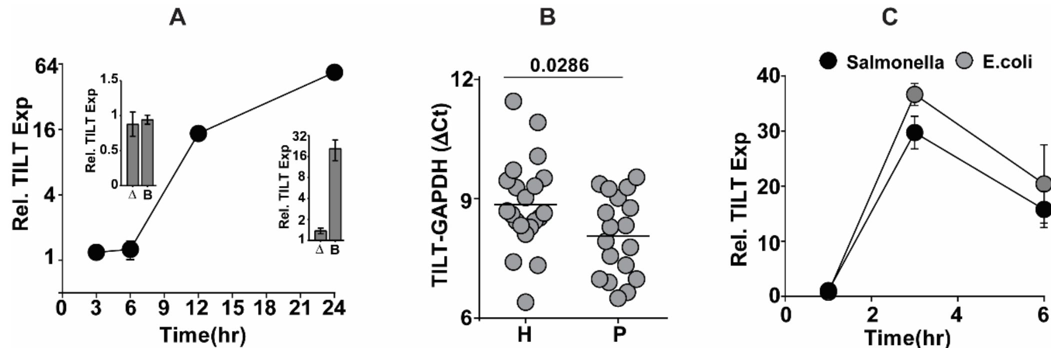

184 **Fig. 3.**

185 **LncRNA- TILT is actively induced in Mtb infected macrophages**

186 A-C, Expression kinetics of TILT in macrophages infected with Mtb at a MOI of 5. At the indicated
187 intervals, RNA prepared from cells was used for analysis by qRT PCR. A- RNA from THP1 cells
188 Inset- at 6 and 24h of infection with Heat killed Mtb (Δ) or BCG. B- from blood of healthy individuals
189 and TB patients; each symbol represents one individual. C- in THP1 macrophages infected with
190 *E. coli*, *S. typhimurium* at a MOI of 5 for the indicated time intervals. Values represented are mean
191 + SEM of triplicate wells of 2-3 independent experiments.

192 **TLR4 signaling acts as the primary stimulus for TILT expression in macrophages**

193 Macrophages, being the primary responders to any infection, are endowed with the capacity to
194 recognize microbial PAMPS via innate receptors like TLRs and activate signaling mechanisms
195 for rapid and robust neutralization of the pathogen. To identify the stimulus involved in activating
196 this lncRNA, we analyzed TILT expression in macrophages stimulated with different TLR ligands
197 (Fig. 4A). Where stimulation of most TLRs resulted in minimal induction, only LPS actively
198 enhanced TILT expression in THP1 cells by ~ 20-fold increase by 6h and a sharp decline to ~9
199 fold by 24h. In response to LPS, the two transcripts- NRIR and TILT showed distinct kinetics of
200 expression. Contrasting with the Mtb induced profile of faster NRIR expression, TILT was strongly

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201 induced as early as 3h (~16 fold) reaching to an excess of 200 times the basal levels by the 6th
202 hour of LPS stimulation and stabilizing thereafter to nearly 100 folds by 48h. In contrast, NRIR,
203 despite any detectable expression by 3h, amplified rapidly to reach ~50 times basal levels with a
204 peak of ~150 fold at 24h and then stabilizing to ~60 fold by 48h of stimulation (Fig. 4B). A complete
205 dependence on TLR4 signaling was observed for expression of TILT in macrophages- treatment
206 with the TLR4 antagonist -CLI095 completely neutralized TILT expression in macrophages in
207 response to LPS, *E. coli* and *S. typhimurium*. In contrast, the effect of CLI0095 was only partial in
208 THP1 infected with Mtb suggesting a TLR4 independent accessory signaling for TLT expression
209 (Fig. 4C).

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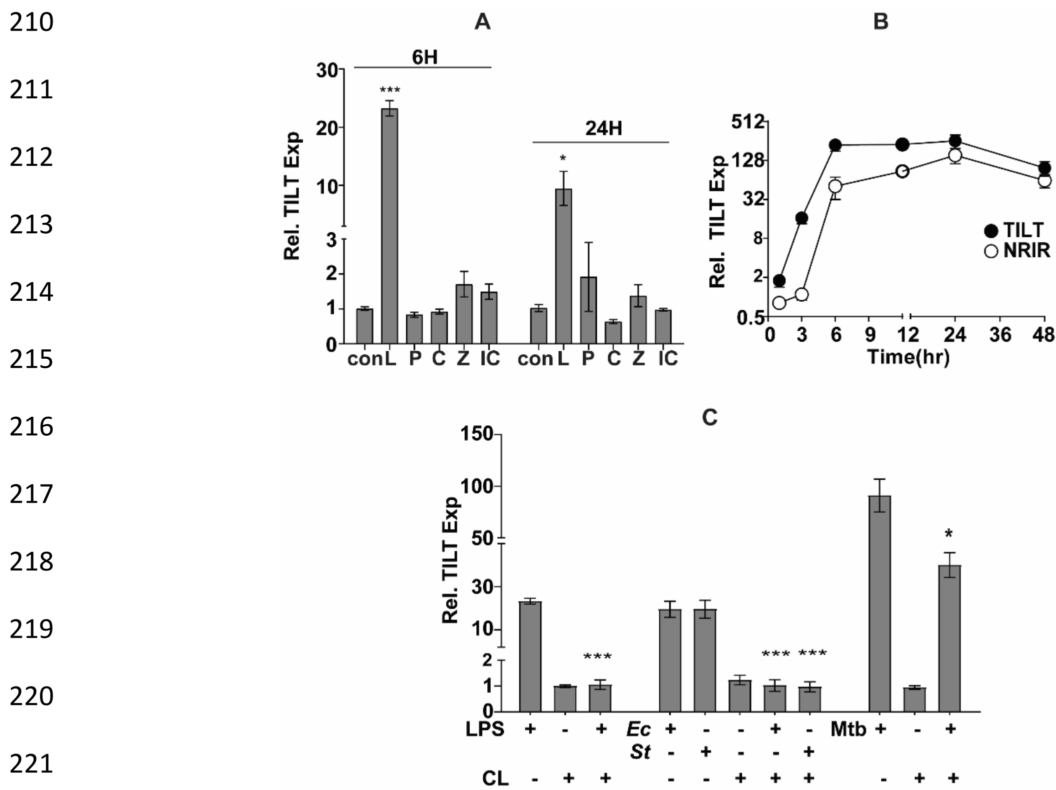

Fig. 4

223 **TILT is induced temporally in response to LPS stimulation in THP1 macrophages.**

224 A- TILT expression in macrophages stimulated with different TLR ligands- L- LPS at 10 ng/ml,
225 P- Peptidoglycan at 20 ng/ml, C- Pam3CSK at 20 ng/ml, Z-Zymosan at 10 μg/ml, IC- poly
226 IC at 2 μg/ml) at 6 and 24h post stimulation. B) Kinetics of TILT and NRIR expression in

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227 macrophages treated with 10ng/ml of LPS. C) TILT expression in macrophages infected
228 with *S. typhi* (3h *p.i.*), *E. coli* (3h *p.i.*), Mtb (24h *p.i.*) or stimulated with LPS (6h *p.s.*) in the
229 presence of the TLR4 inhibitor CLI095 (3 μM). At the indicated intervals RNA prepared
230 from cells was used for analysis by qRT PCR and normalized to GAPDH. Relative
231 expression at any given time w.r.t unstimulated is depicted as mean $\pm$ SEM for triplicate
232 assays of 2-3 independent experiments.
233

234 **DISCUSSION**

235 Macrophages have been shown to successfully respond to numerous stimuli including infections
236 and initiate response profiles for control of the pathogen (Weiss and Schaible 2015). Over several
237 decades of research, the landscape of stimuli driven protein coding expression patterns have
238 been realized in high detail (Roy et al. 2018). It is now possible to portray the layout of protein/
239 pathway induction profiles in host macrophages as a response to infection with several bacterial
240 pathogens (Denzer et al. 2020). The discovery of several non-protein coding transcripts as key
241 regulators has only supplemented another layer to the multi-scale pyramid of the macrophage
242 response kinetics (Aune and Spurlock 2016; Duval et al. 2017; Zur Bruegge et al. 2017; Ma et al.
243 2019; Ahmad et al. 2020). In the last few years, several non-coding transcripts and regulatory
244 networks have been identified with important regulatory roles in infection with the help of powerful
245 omics-untargeted / specific-targeted approaches (Moon et al. 2014; Liu et al. 2018; Menard et al.
246 2018; Fan et al. 2019; Liu et al. 2019).
247 By using simple molecular techniques, we have identified novel non coding transcripts (TILT1-3)
248 encompassing a previously identified lncRNA (NRIR) and a mitochondrial resident gene
249 (CMPK2). Recently, by using high throughput Capture Long Seq (CLS), GENCODE consortium
250 have re annotated lncRNAs population in human and mouse tissues identifying several noncoding
251 transcripts (Lagarde, 2017 #77}. We demonstrate the presence of multiple transcripts comprising
252 of different exons from the NRIR and CMPK2 genomic locus implying a higher degree of splicing

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253 based regulation of gene expression in this region. Our finding of TILT in human blood cells of a
254 normal individual also argues for the presence of these transcripts in myeloid cell populations.
255 Surprisingly, this arrangement of NRIR with CMPK2 is only observed in human cells and is absent
256 from murine cells suggestive of a unique regulatory network to fine tune macrophage response
257 kinetics (Breschi et al. 2017).

258 Despite a differential amplitude and kinetics of expression of the two non-coding transcripts (NRIR
259 and TILT) in response to different stimuli, the rapid and sustained levels of expression over 24-
260 48h of stimulus highlights the importance of this locus in the response kinetics of macrophages.
261 Increased expression of TILT in sera of active TB patients only signifies the importance of this
262 response in TB infections, coupled to the enhanced expression of TILT in response to infection
263 with *E. coli* and Salmonella argues for an important regulatory circuit in macrophage response to
264 infection. Further the strong induction of TILT with LPS stimulation earlier than the expression of
265 NRIR is suggestive of a differential control of expression. While we did not find promoter like
266 element different from the annotated promoter of CMPK2 (Data not shown), separate promoter
267 like elements were observed upstream of NRIR indicative of an uncoupled expression of this
268 lncRNA from CMPK2 and TILT. Abrogation of TILT expression following treatment with CLI095 in
269 LPS stimulated, in *E. coli*, Salmonella infections only substantiates the absolute requirement of
270 TLR4 mediated signaling for TILT expression in macrophages.

271 In sharp contrast to NRIR, expression of TILT was more protracted and reduced in comparison in
272 response to Mtb infection. Moreover, treatment of Mtb infected cells with CLI095 only partially
273 reduced the expression of TILT in macrophages. It is well established that macrophage response
274 to Mtb is not primarily dependent on TLR4 signaling with combinatorial requirement for other TLR
275 (TLR2), NLR, RLR mediated signaling rather than predominant signaling axis (Kleinnijenhuis et
276 al. 2011; Faridgohar and Nikoueinejad 2017). In fact, mice lacking multiple TLR did not suffer
277 from increased susceptibility to Mtb infection (Nguyen et al. 2020) again corroborating the multiple
278 axis of macrophage stimulation by Mtb leading to the enormous redundancy and plasticity of the

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279 macrophage responses. Identifying the regulatory circuit and the consequence of this control in
280 macrophage/ immune cell function would provide important insights into the diversity and
281 complexity of mammalian innate responses.

282 **Material and Methods**

283 **Bacterial Strains and Growth Conditions-**

284 *Mycobacterium tuberculosis* strain Erdman was grown in 7H9 Middlebrook enriched with
285 Middlebrook ADC (BD Biosciences, USA). *E. coli* and *Salmonella typhimurium* were grown in LB
286 media (BD Biosciences, USA) at 37°C.

287 **Macrophage culture and Infection**

288 RPMI 1640-GlutaMAX (Himedia laboratories, Mumbai, India) supplemented with 10% fetal bovine
289 serum (Himedia laboratories, Mumbai, India,) and 1mM sodium pyruvate (Himedia laboratories,
290 Mumbai, India) was used to culture THP-1 monocytes. Differentiation of THP1 monocytes to
291 macrophages was done using 100nM phorbol myristate acetate (PMA) (Sigma Aldrich, USA).
292 Mtb, *E. coli* and *Salmonella typhimurium* were grown to mid-log phase at 37°C, washed twice with
293 phosphate buffered saline (PBS) containing 0.05% Tween80, finally suspended in PBS and
294 centrifuged at 800 rpm for 10 min to get a uniform single cell suspension. This uniform single cell
295 suspension was diluted with complete RPMI 1640 to the required cell density and then used to
296 infect the differentiated THP-1 macrophages at multiplicity of infection (MOI) of 5. At different time
297 intervals, cells were harvested in RNAzol (Sigma Aldrich, USA) for analysis. LPS, CLI095, poly
298 I:C, and Pam3CSK2 were purchased from (InvivoGen, Toulouse, France). Zymosan (Sigma
299 Aldrich, USA) was used according to the concentration mentioned.

300 **RNA isolation and qRT-PCR**

301 RNA isolation was done as per standard RNAzol protocol. cDNA synthesis was performed from
302 1µg RNA using verso cDNA synthesis kit (Thermo Scientific, USA). The expression level was
303 checked by DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific, USA) in Roche LC480
304 system. *Gapdh* was used as internal control. Ct values were normalized with uninfected control.

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305 **Genome locus and PCR**

306 Genome locus was analyzed using UCSC web (https://genome.ucsc.edu/.)

307 **Whole blood collection**

308 Confirmed cases of pulmonary TB patients between ages of 5 and 15 years were included in the
309 study with prior consent as per the Institutional ethical committee guidelines. BCG vaccination,
310 primary or secondary infection and single or co-infection status were recorded. HIV positive and
311 any non-respiratory major disease patients were excluded from the study. The control samples
312 were obtained from individuals without any overt clinical manifestation of disease. 2-3 ml blood
313 was collected in RNAgard blood tubes (Biomatrica, USA) and stored in -80°C until RNA
314 precipitation. RNA was isolated from frozen blood samples as per the manufacturer's
315 recommendations.

316 **Graphs**

317 Graphs were generated using Graphpad prism or R. Student t test was used for statistical analysis
318 of the data.

319 **Table 1: List of primers used in the study**

Name	Primer Sequence
1	CCACCCCCACGAAGAAATTATATATC
2	GTTAGAGGTGTCTGCTGCAATAATC
3	CCAGGTTGTTGCCATCGAAG
4	CAAGAGGGTGGTGACTTTAAGAG
5	CTGGAATGACCCCTGACATC
6	GGTGGGATTGAACTGAACAG

320

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323 **Author Contributions**: PA, VR were involved in conceptualizing and design of the work, MS and
324 RL were instrumental in sampling of blood from TB patients, the work was performed by PA and
325 manuscript was written by VR and PA.

326 **Conflict of interest**: The authors do not have any competing interests.

327

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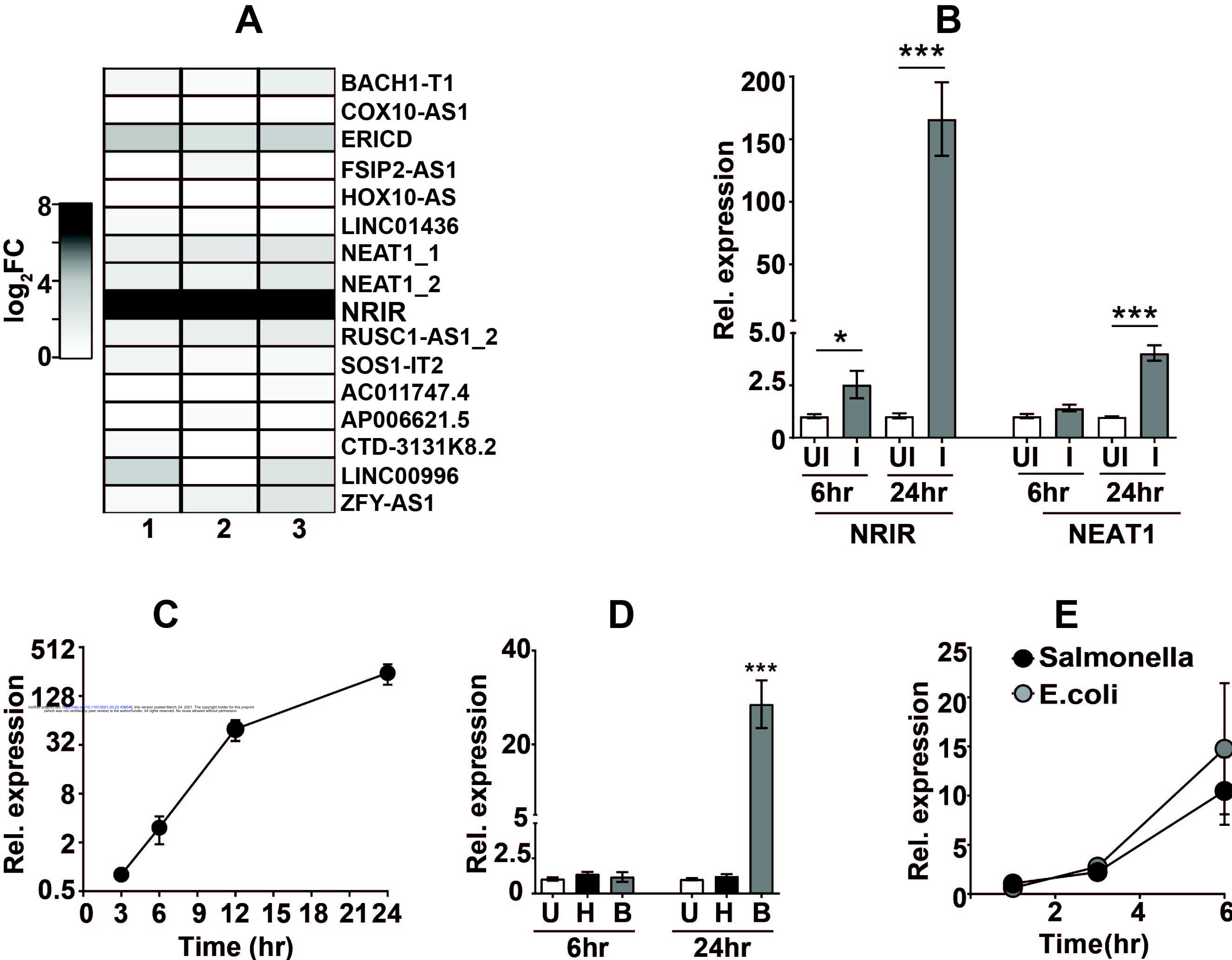

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Fig. 1

A

1 5 6 3 4

2

Scale 20 kb hg38

chr2: 6,820,000 6,825,000 6,830,000 6,835,000 6,840,000 6,845,000 6,850,000 6,855,000 6,860,000 6,865,000

NRIR-201

NRIR-202

3 4

CMPK2-203

1 2

CMPK2-201

5 6

CMPK2-202

Chimp

Orangutan

Rhesus

Green monkey

Mouse

Rat

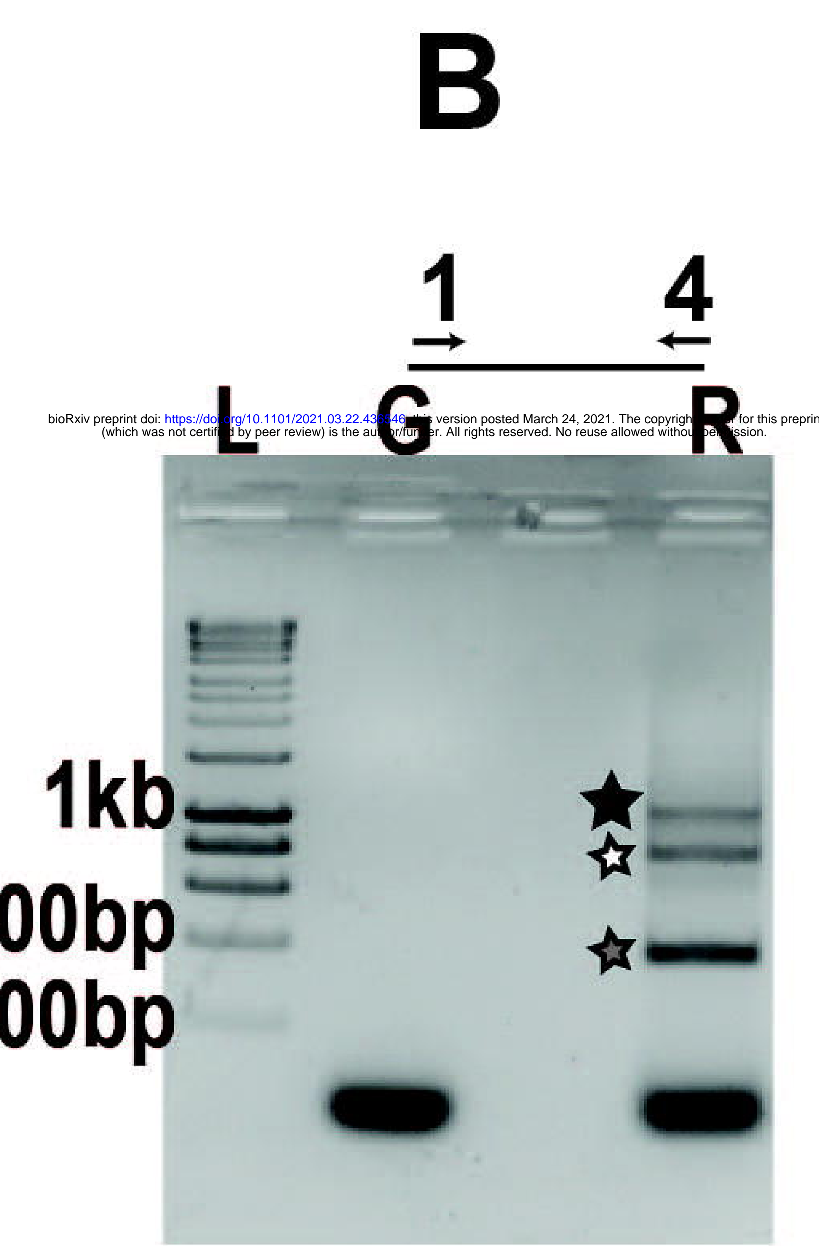
Guineapig

Cow

Domestic goat

Zebrafish

B



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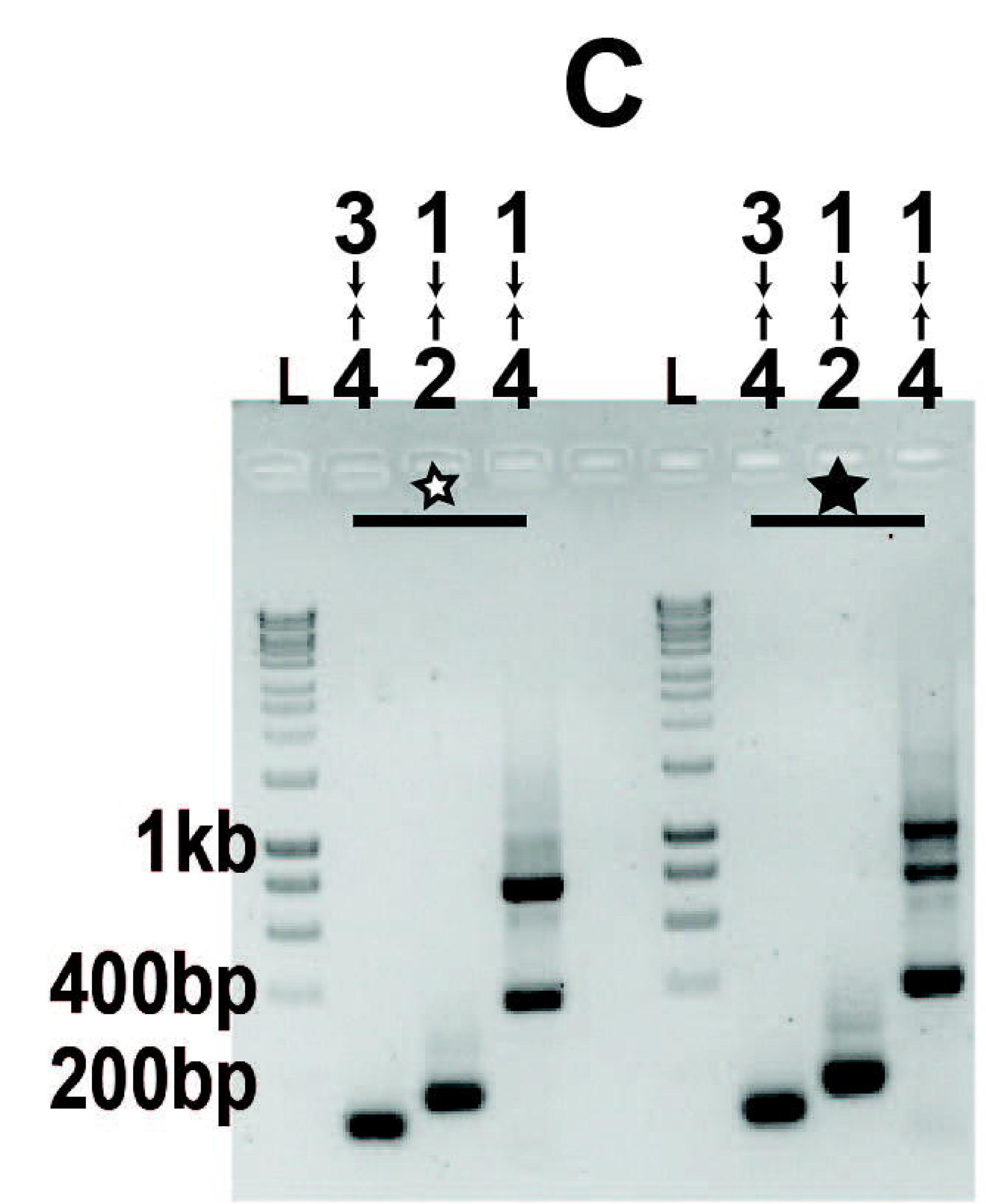

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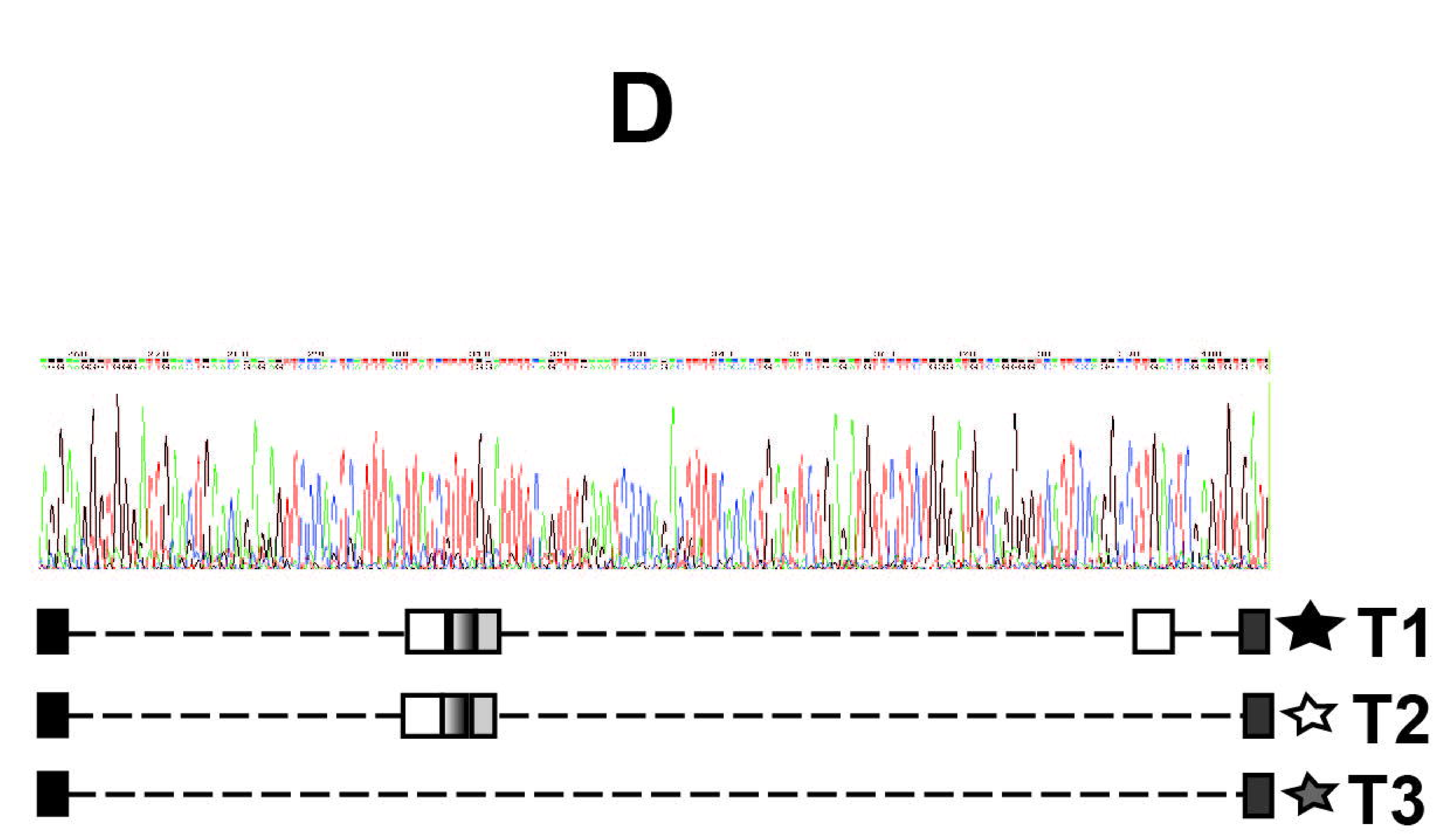

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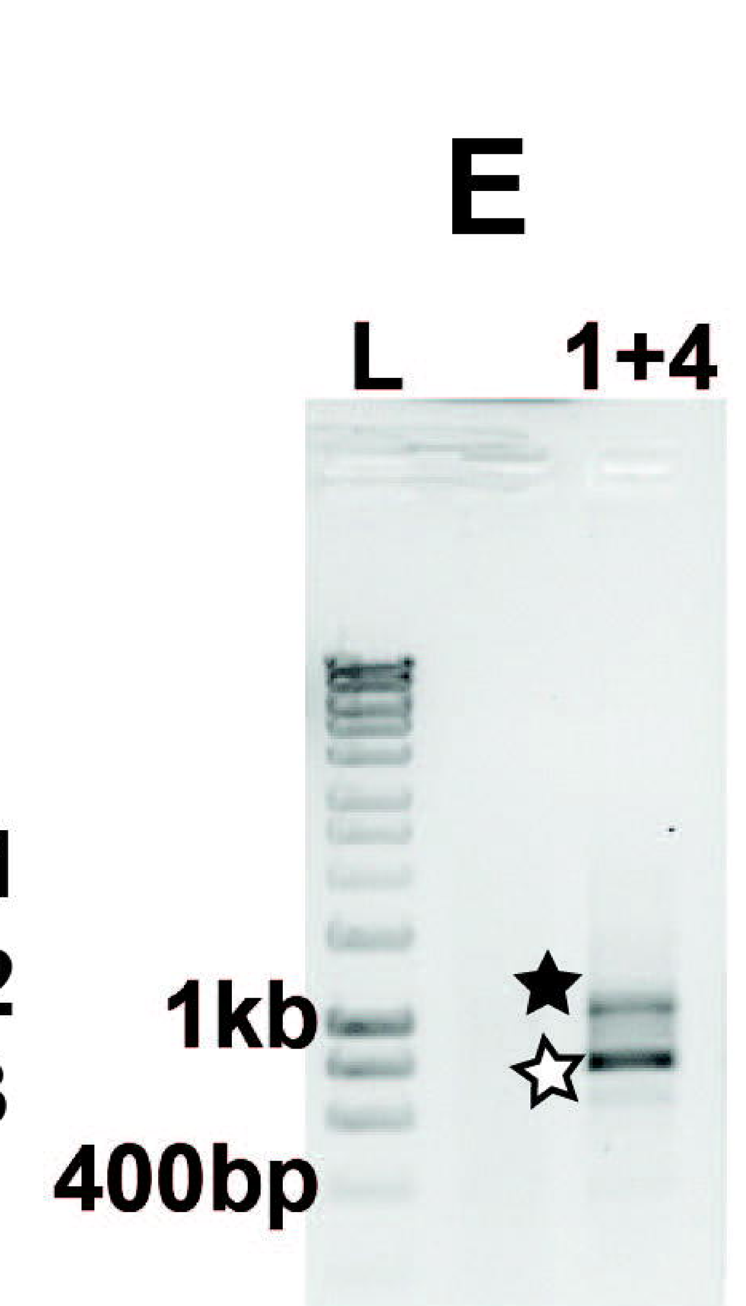

Fig. 2

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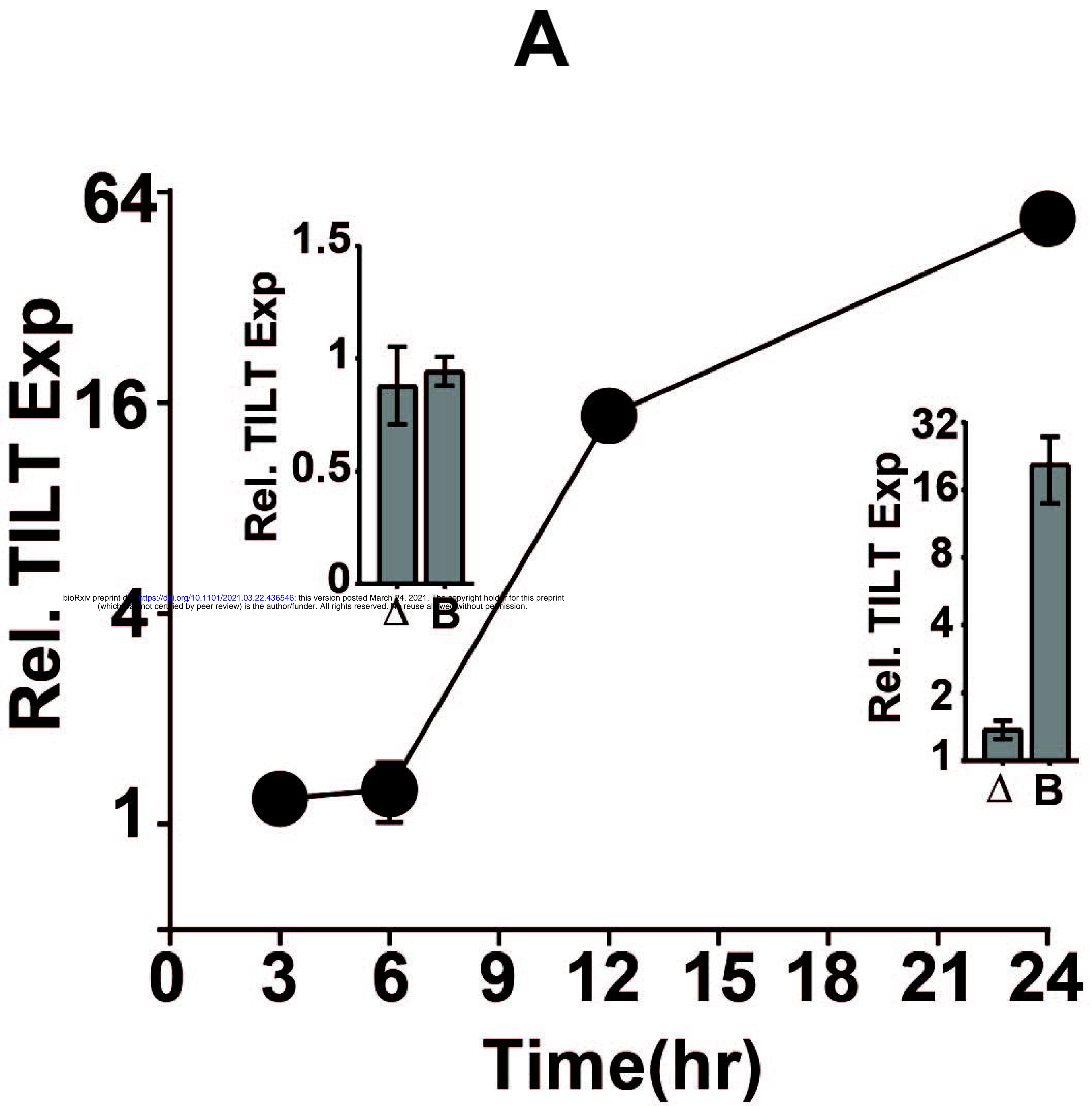

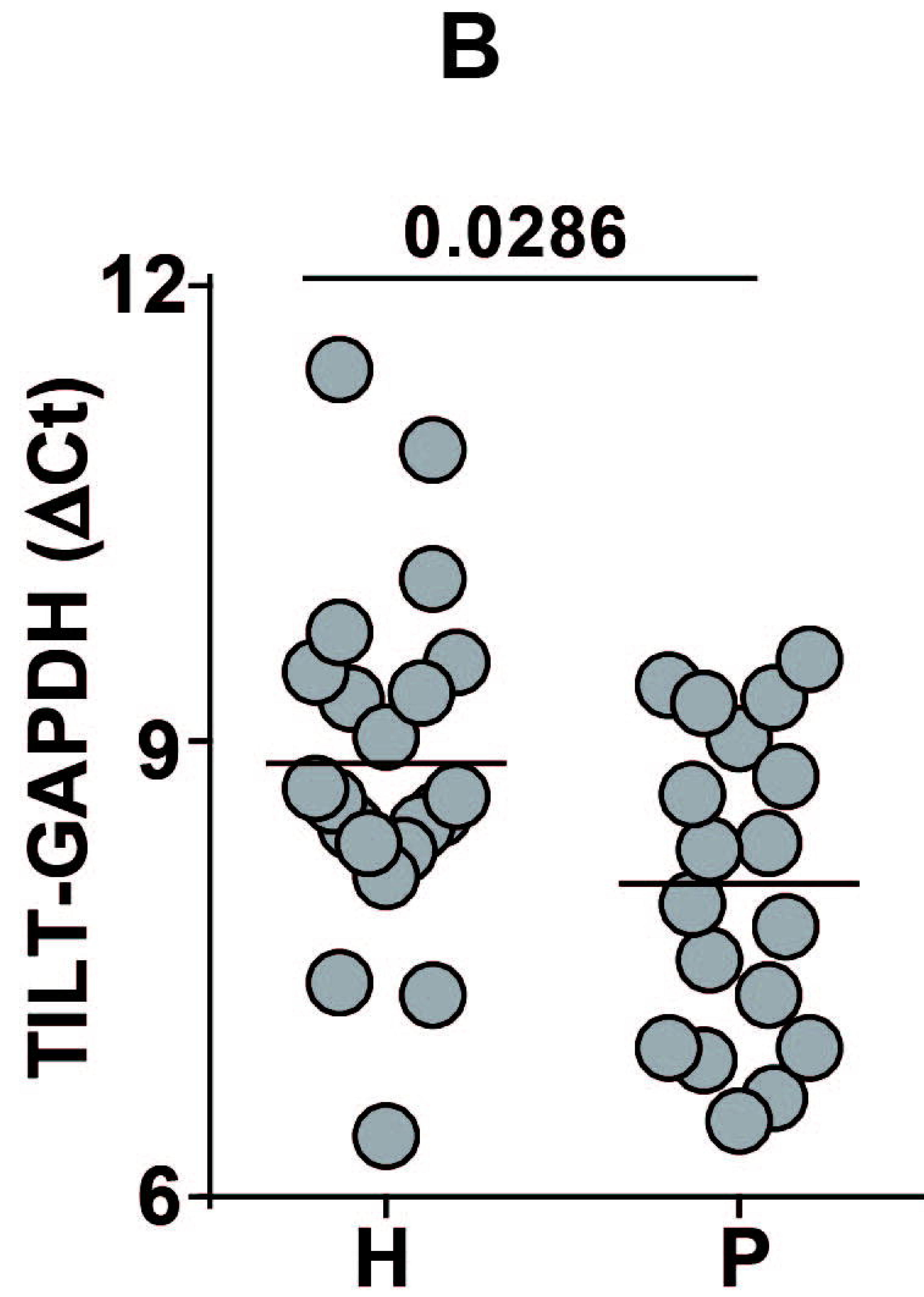

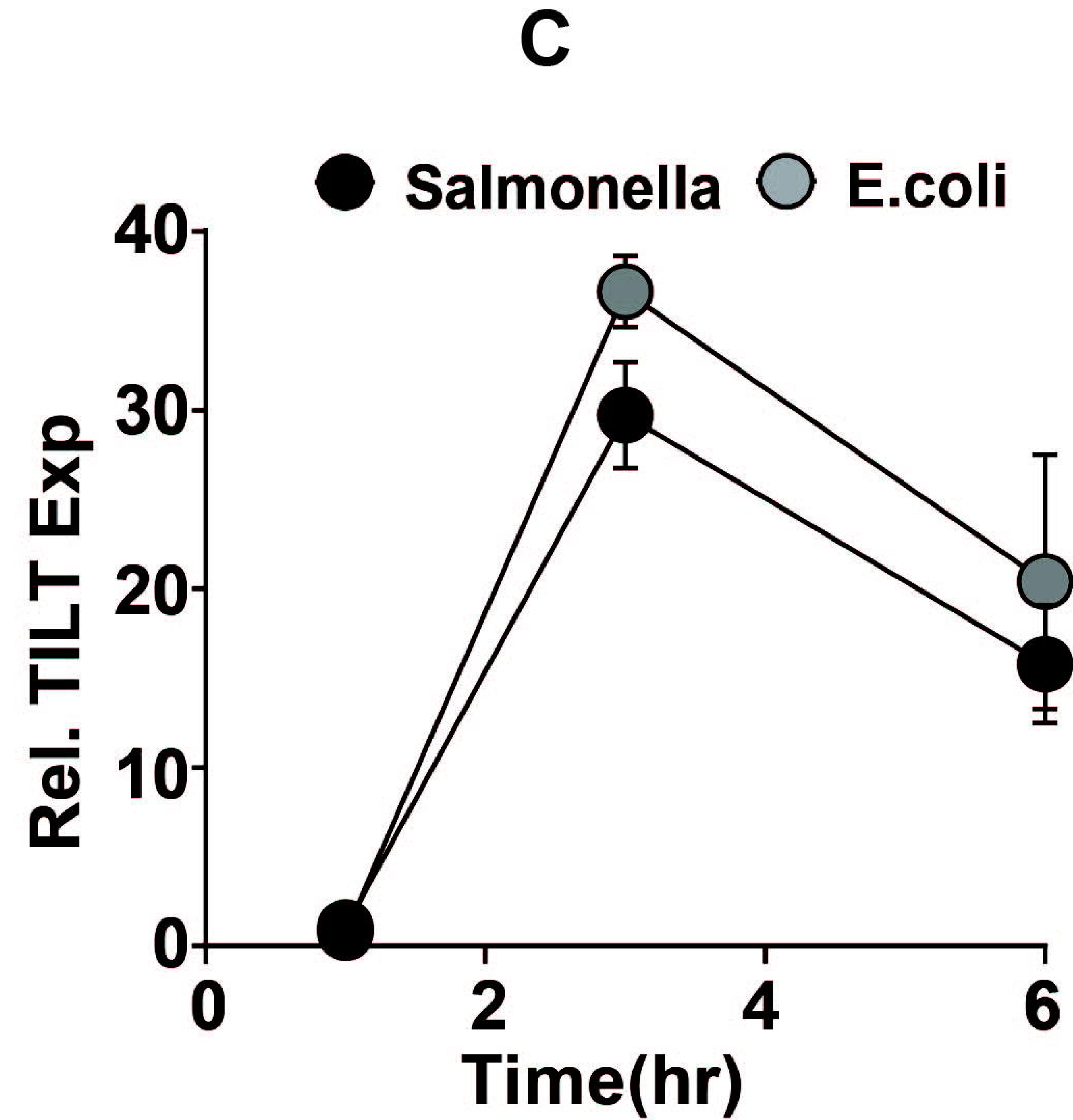

Fig. 3

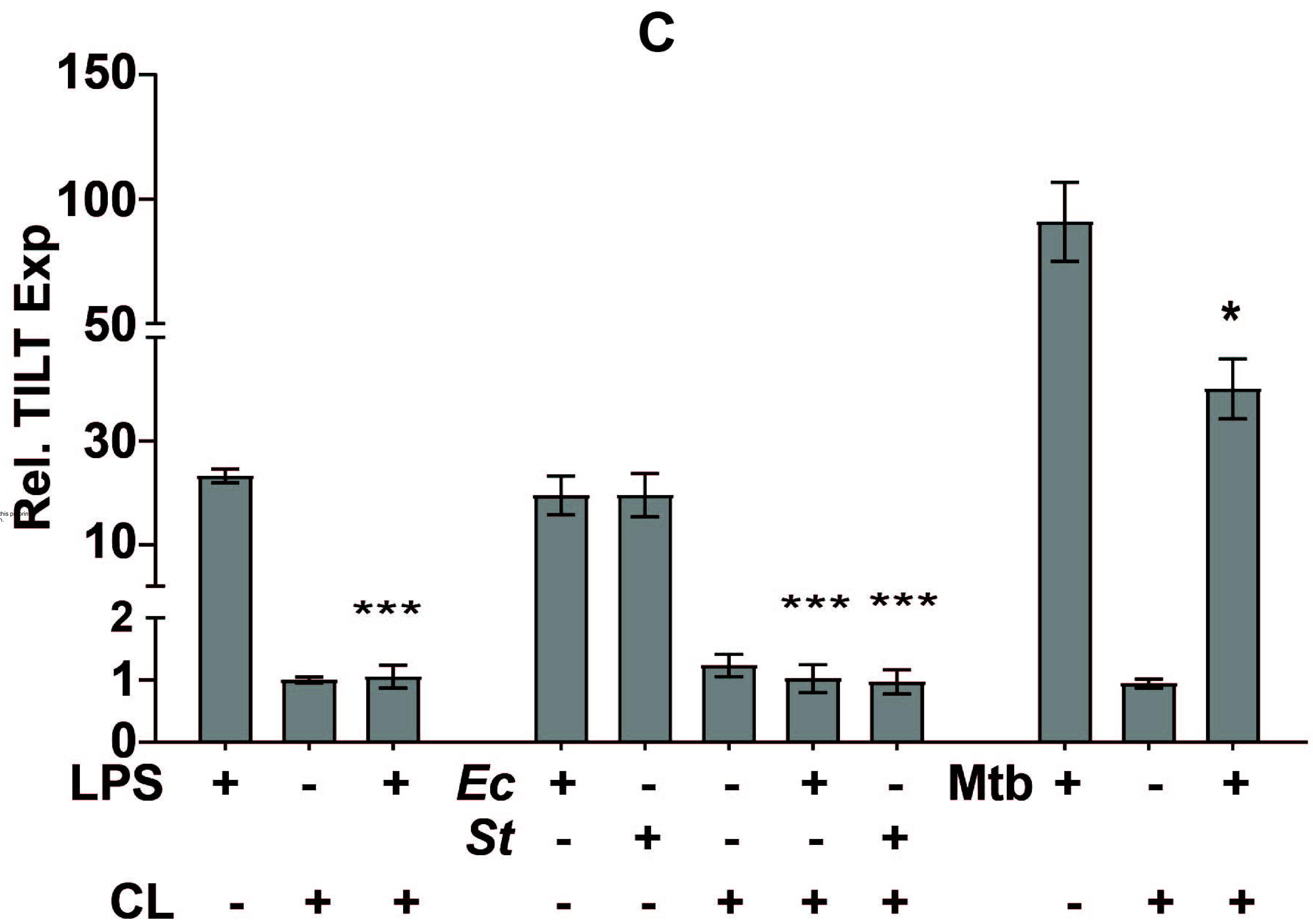

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Fig. 4